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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, A61K 39/00, 35/14, C07K 14/705</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/26271</b> <b>(43) International Publication Date:</b> 29 August 1996 (29.08.96)
<b>(21) International Application Number:</b> PCT/US96/02156 <b>(22) International Filing Date:</b> 13 February 1996 (13.02.96)  <b>(30) Priority Data:</b> 08/396,385      22 February 1995 (22.02.95)      US  <b>(71) Applicants:</b> THERION BIOLOGICS CORPORATION [US/US]; 76 Rogers Street, Cambridge, MA 02142 (US). UNITED STATES GOVERNEMENT, represented by THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).  <b>(72) Inventors:</b> SCHLOM, Jeffrey; 10301 Sorrel Avenue, Potomac, MD 20854 (US). PANICALI, Dennis; 114 Nonset Path, Acton, MA 01720 (US). TSANG, Kwong, Y.; 5420 York Lane, Bethesda, MD 20814 (US).  <b>(74) Agents:</b> BRONSTEIN, Sewall, P. et al.; Dike, Bronstein, Roberts & Cushman, 130 Water Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> GENERATION OF HUMAN CYTOTOXIC T-CELLS SPECIFIC FOR CARCINOMA SELF-ASSOCIATED ANTIGENS AND USES THEREOF  <b>(57) Abstract</b> <p>We have discovered that by using a recombinant DNA viral vector, preferably a pox virus vector having at least one insertion site containing a DNA segment encoding the carcinoma self-associated antigen, or a cytotoxic T-cell eliciting epitope thereof, operablylinked to a promoter capable of expression in the host, human cytotoxic T-cells specific for the carcinoma self-associated antigens can be produced. The method preferably comprises introducing a sufficient amount of the recombinant pox virus vector into a host to stimulate production of cytotoxic T-cells, and contacting the host with additional antigen at periodic intervals thereafter. The additional antigen may be added by using a second pox virus vector from a different pox genus. In another embodiment, additional antigen is added by contacting the host with antigen. The antigen may be formulated with an adjuvant or in a liposomal formulation. The T-cells can be isolated. The number of T-cells can be expanded by contacting the isolated cytotoxic T-cells alternately with the carcinoma self-associated antigen or an epitope thereof and IL-2. The isolated T-cells can be used in a method for treating a host having a tumor expressing a carcinoma self-associated antigen comprising introducing cytotoxic T-cells specific for the antigen to the host and at at least one periodic interval thereafter introducing to the host a T-cell eliciting epitope of the carcinoma self-associated antigen.</p>		

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## **GENERATION OF HUMAN CYTOTOXIC T-CELLS SPECIFIC FOR CARCINOMA SELF-ASSOCIATED ANTIGENS AND USES THEREOF**

### **FIELD OF THE INVENTION**

The present invention relates generally to generation of human cytotoxic T-cells specific for carcinoma self-associated antigens and uses thereof, for example in epitope mapping and adoptive cell therapy.

5

### **BACKGROUND OF THE INVENTION**

A number of approaches have been utilized in treating malignant growth of tissue cells in humans such as cancers. While the various approaches identified have frequently been successful in treating a particular cancer, one difficulty has been that there are numerous different cancers. Thus, drugs that treat one type of cancer, often are ineffective against a different type of cancer.

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One method that attempts to overcome the difficulties resulting from the differences in cancers is immunotherapy. By such an approach, the immune system can select and create the method of treating a specific cancer. Being able to select and/or identify antigens including specific epitopes that can be used in such an approach is extremely important.

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Specific peptides that bind human MHC molecules have now been identified for melanoma associated antigens. Darrow, T.L., et al. *J Immunol* 142: 3329-3335, (1989); Hom, S.S., et al. *J Immunother*

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10: 153-164, (1991); Cox, A.L., et al. *Science* 264: 716-719 (1994); Olive, D., et al. *Cancer Vaccine Symposium*. Cancer Research Institute. Oct. 3-4 (1994). MHC class I and/or class II peptide complexes have been reported as being recognized by human T-cells. The ability to  
5 activate T-cells by cytokines or co-stimulatory molecules is thus extremely important.

The identification of human carcinoma associated antigens and epitopes that might be recognized by human T-cells is also currently  
10 under active investigation. Molecules such as prostate specific antigen (PSA), [Oesterling, J.E., *J Urol* 145: 907-923, (1991); Peace, D.J., et al *Cancer Vaccine Symposium*. Cancer Research Institute. Oct. 3-5 (1994)], neu/c-erbB2 [Fisk, B., et al. *Int J Oncology* 5: 51-63 (1994)]  
MUC-1 [Ioannides, C.G., et al. *J Immunol* 151: 3693-3703, (1993)]  
15 point mutated ras [Tsang, K.Y., et al. *Vaccine Research* (in press); Jung, S., et al. *J Exp Med* 173: 273-276 (1991); Fenton, S., et al. *J Natl Cancer Inst* 85: 1294-1302, (1993)] point mutated p53 [Houbliers, J.G.A., et al *Eur J Immunol* 23: 2072-2077, (1993)] and  
carcinoembryonic antigen (CEA) [Kantor, J., et al. *J Natl Cancer Inst*  
20 84: 1084-1091, (1992); Kantor, J., et al *Cancer Res* 52: 6917-6925, (1992); Ras, E., et al. European Immunology meeting, Barcelona, June (1994)] are among such candidates. One difficulty has been that many of these antigens are normal self-antigens, which therefore are not  
25 expected to trigger an immune response of the type needed for therapeutic approaches.

For example, while human CEA is extensively expressed on the vast majority of human colorectal, gastric and pancreatic carcinomas

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as well as approximately 50% of breast cancers and 70% of non-small cell lung cancers [Thompson, J.A., et al. *J Clin Lab Anal* 5: 344-366, (1991)], CEA is also expressed at least to some extent on normal colon epithelium and in some fetal tissue. The CEA gene has been  
5 sequenced and shown to be part of the human immunoglobulin gene superfamily, and thus shares some homology with other molecules found on normal human tissues. Thompson, J.A., et al. *J Clin Lab Anal* 5: 344-366, (1991); Oikawa, S., et al. *Biochem Biophys Res Commun* 144: 634-642, (1987). At the amino acid level, CEA shares  
10 approximately 70% homology with NCA (non-specific cross reacting antigen) which is found on normal granulocytes. Thompson, J.A., et al. *supra*.

However, the immunogenicity of CEA in normal humans or  
15 cancer patients is at best suspect. Although several papers claim antibodies to CEA in patients, [Staab, H.J., et al *Br J Cancer* 42: 26-33, (1980); Mavligit, G.M., et al. *Cancer* (Phila) 52: 146-149, (1983)] others claim these observations are artifacts [Collatz, E., et al *Int J Cancer* 8: 298-303, (1971); Chester, K.A., et al *Clin Exp Immunol* 58:  
20 685-693, (1984); Ura, Y., et al. *Cancer Lett* 24: 283-295, (1985)]. No reports of the presence or absence of T-cell responses to CEA exist.

There are two types of immune response --antigen specific responses producing antibodies, and cell specific responses eliciting  
25 cytotoxic T-cells.

Improved methods for eliciting an immune response to self-antigen would be extremely useful.

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For example, cytotoxic T-cells elicited by a self-antigen can be used in somatic cell therapy, in identifying epitopes and small peptides that induce a cytotoxic T-cell response and in drug assays for compounds that enhance a specific cytotoxic T-cell response.

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The availability of human cytotoxic T-cells specific for carcinoma self-associated antigens also allow for mapping of the epitopes recognized by the T-cells. These epitopes can in turn be used to prime or boost the immune-system to expand the T-cell population either *in vivo* or *in vitro*. The *in vitro* cells as aforesaid can then be transferred back into a patient as is currently done in TIL cell therapy and used to treat a tumor expressing the antigen.

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#### SUMMARY OF THE INVENTION

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We have discovered that by using a recombinant DNA viral vector, preferably a pox virus vector having at least one insertion site containing a DNA segment encoding the carcinoma self-associated antigen, or a cytotoxic T-cell eliciting epitope thereof, operably linked to a promoter capable of expression in the host, human cytotoxic T-cells specific for the carcinoma self-associated antigens can be produced. The method preferably comprises introducing a sufficient amount of the recombinant pox virus vector into a host to stimulate production of cytotoxic T-cells, and contacting the host with additional antigen at periodic intervals thereafter. The additional antigen may be added by using a second pox virus vector from a different pox genus. In another embodiment, additional antigen is added by contacting the host with antigen. The antigen may be formulated with an adjuvant or in a liposomal formulation.

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We have also discovered that human cytotoxic T-cells specific for carcinoma self-associated antigens can be produced using a cytotoxic T-cell eliciting epitope of the carcinoma self-associated antigen. The method preferably comprises introducing the T-cell eliciting epitope into a host to stimulate production of cytotoxic T-cells. If necessary, in order to boost production of the cytotoxic T-cells, at periodic intervals thereafter, the host is contacted with additional T-cell eliciting epitope. The epitope may be formulated with adjuvant or may be in a liposomal formulation. Alternatively, additional T-cell eliciting epitope may be added using a pox virus vector.

Carcinoma self-associated antigens include, for example, carcinoembryonic antigen (CEA), prostate specific antigen (PSA), TAG-72, IL-2r and *neu/c-erbB-2*. CEA is preferred.

Cytotoxic T-cell eliciting epitopes of the carcinoma self-associated antigens may also be used. For CEA, preferred epitopes comprise peptides represented in the Sequence Listing as SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.

The pox virus is preferably selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus. Preferred orthopox include vaccinia, rabbitpox and racoon pox. Preferred avipox includes fowlpox, canary pox and pigeon pox. A more preferred avipox is fowlpox. The preferred suipox is swinepox.

Vaccinia viral vectors may elicit a strong antibody response, thus while numerous boosts with vaccinia vectors are possible, its repeated



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use is not preferred. We have discovered that by using pox from different genera to boost, this sensitivity problem can be minimized. In accordance with the present invention, in order to avoid such problems, preferably, when the first or initial pox virus vector is vaccinia, the second and subsequent pox virus vectors are selected from the pox viruses from a different genus such as suipox, avipox, capripox other than vaccinia.

Adjuvants include, for example, RIBI Detox, Q521, and incomplete Freund's adjuvant. Liposomal formulations can also be used.

Human cytotoxic T-cells specific for a carcinoma self-associated antigens produced in accordance with the present invention can be isolated from a human host. These cells can be used in drug assays, used to map cytotoxic T-cell eliciting antigen epitopes or in adoptive cell therapy.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and Figure 1B show cytotoxicity of a T-cell lines [designated V24T (Figure 1A) and V6T (Figure 1B)] derived from patients immunized with rV-CEA; and induced by CEA CAP-1 peptide. CTL activity was determined in a 18hr <sup>111</sup>In-release assay using T2 cells as target incubated with CAP-1 peptide (50μg/ml).

Figure 2 depicts secretion of TNF-alpha by T-VAC24 cell line in response to stimulation of autologous B-cells pulsed with CEA peptide. The supernant was screened for TNF-alpha secretion using a ELSIA

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during a 3 day incubation of V24T-cells with autologous B cells and CEA peptide (50  $\mu$ g/ml).

#### DETAILED DESCRIPTION OF THE INVENTION

5           We have induced T-cell immunity to CEA in carcinoma patients by placing the CEA gene into a recombinant viral vector, i.e, a pox vector such as vaccinia virus. Vaccinia is a preferred vector for several reasons. Among these are: (a) its wide use in humans in the eradication of smallpox; (b) its ability to infect a wide range of cells,  
10           including professional antigen presenting cells, and express the inserted gene product in a manner that it has the potential to be processed in the context of class I and/or class II MHC molecules; and (c) animal model studies have shown that the use of a recombinant human CEA vaccinia virus (designated rV-CEA) is superior to the use of soluble  
15           CEA in the induction of anti-tumor effects on established CEA expressing tumors. Kantor, J., et al. *J Natl Cancer Inst* 84: 1084-1091, (1992). These findings correlated with the appearance of CEA specific CTLs in rV-CEA inoculated animals. Kantor, J., et al., *supra*.

20           Animal model studies demonstrate that rV-CEA can indeed infect mammalian cells *in vivo* to a level as to induce immune responses and demonstrate lack of toxicity.

25           rV-CEA has also been administered to rhesus monkeys and has been shown to induce CEA-specific T-cell responses with no toxicity. Kantor, J., et al *Cancer Res* 52: 6917-6925, (1992).

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Cytotoxic T-cell lines can also be generated using a cytotoxic T-cell eliciting epitope of a carcinoma self-associated antigen.

5 As used herein a carcinoma self-associated antigen refers to an antigen that is native to the animal and associated with malignant tumors. Preferred self-associated antigens include, for example, CEA, PSA, *neu/c-erbB-2*, TAG-72 and IL-2r. More, preferably, the antigens are CEA and PSA. Even more preferably CEA.

#### 10 Viral vector

Basic techniques for preparing recombinant DNA viruses containing a heterologous DNA sequence encoding the carcinoma self-associated antigen or cytotoxic T-cell eliciting epitope are known to the skilled artisan and involve, for example, homologous recombination  
15 between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982)). For example, recombinant viral vectors such as a pox viral vector can be used in delivering the gene. The vector can be constructed for  
20 example by steps known in the art, e.g. analogous to the methods for creating synthetic recombinants of the fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference. Other techniques include using a unique restriction endonuclease site that is naturally present or artificially inserted in the  
25 parental viral vector to insert the heterologous DNA.

Pox viruses useful in practicing the present invention include orthopox, suipox, avipox and capripoxvirus.

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Orthopox include vaccinia and ectomelia, raccoon pox. The preferred orthopox is vaccinia.

5           Avipox includes fowlpox, canary pox and pigeon pox. The preferred avipox is fowlpox.

A preferred suipox is swinepox.

10           Other viral vectors that can be used include herpes virus, retroviruses, polio, and adenoviruses.

15           For example, the DNA gene sequence to be inserted into the virus can be placed into a donor plasmid, e.g., an *E. coli* plasmid construct, into which DNA homologous to a section of DNA such as that of the insertion site of the poxvirus where the DNA is to be inserted has been inserted. Separately the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking  
20           a region of pox DNA which is the desired insertion region. With a parental pox viral vector, a pox promoter is used. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria and isolated. Preferably, the plasmid also contains an origin of replication such as the *E. coli* origin of replication, and a marker such  
25           as an antibiotic resistance gene for selection and propagation in *E. coli*.

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g., chick embryo

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fibroblasts, along with the parental virus, e.g., poxvirus.

Recombination between homologous pox DNA in the plasmid and the viral genome respectively results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome, at a site which does not affect virus viability.

As noted above, the gene is inserted into a region (insertion region), in the virus which does not affect virus viability of the resultant recombinant virus. The skilled artisan can readily identify such regions in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, the TK gene has been found in all pox virus genomes examined [leporipoxvirus: Upton, et al., *J. Virology*, 60:920 (1986) ( Shope fibroma virus); capripoxvirus: Gershon, et al., *J. Gen. Virol.*, 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, et al., *J. Virol.*, 46:530 (1983) (vaccinia); Esposito, et al., *Virology*, 135:561 (1984) (monkeypox and variola virus); Hruby, et al., *PNAS*, 80:3411 (1983) (vaccinia); Kilpatrick, et al., *Virology*, 143:399 (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al., *J. Gen. Virol.* 69:1275 (1988) (fowlpox); Boyle, et al., *Virology*, 156:355 (1987) (fowlpox); Schnitzlein, et al., *J. Virological Methods*, 20:341 (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., *J. Gen. Virol.* 73:3235-3240 (1992)].

In vaccinia, in addition to the TK region, other insertion regions include, for example, HindIII M.

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In fowlpox, in addition to the TK region, other insertion regions include, for example, BamHI J [Jenkins, et al., *AIDS Research and Human Retroviruses* 7:991-998 (1991)] the *EcoRI-HindIII* fragment, BamHI fragment, *EcoRV-HindIII* fragment, BamHI fragment and the HindIII fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., *J. of Virol.* 67:3069-3076 (1993); Taylor, et al., *Vaccine* 6:497-503 (1988); Spehner, et al., (1990) and Boursnell, et al., *J. of Gen. Virol.* 71:621-628 (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region.

In addition to the requirement that the gene be inserted into an insertion region, successful expression of the inserted gene by the modified poxvirus requires the presence of a promoter operably linked to the desired gene, i.e., in the proper relationship to the inserted gene. The promoter must be placed so that it is located upstream from the gene to be expressed. Promoters are well known in the art and can readily be selected depending on the host and the cell type you wish to target. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, 40K or fowlpox promoters such as FPV C1A. Enhancer elements can also be used in combination to increase the level of expression. Furthermore, the use of inducible promoters, which are also well known in the art, in some embodiments are preferred.

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**Generation Cytotoxic T-Cells**

Cytotoxic T-Cells specific for the desired self-associated antigen can be generated by administering between about  $10^5$ - $10^9$  pfu of the recombinant pox virus, constructed as discussed above to a host. The preferred host is a human. However, a transgenic mammal, e.g., mouse, having a human-like immune system may also be used. At at least one interval thereafter, which is preferably one to three months later, the immune response is boosted by administering additional antigen or a T-cell stimulating epitope thereof to the host. More preferably there is at least a second "boost" preferably one to three months after the first boost. The antigen may preferably be administered using a second pox virus vector from a different pox genera, or may be administered directly using, for example, an adjuvant or liposome. Cytokines, e.g., IL-2, or co-stimulatory molecules, e.g., B7.1, B7.2, may be used as biologic adjuvants and can be administered systemically to the host or co-administered via insertion of the genes encoding the molecules into the recombinant pox vector.

Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 and incomplete Freund's adjuvant.

Cytotoxic T-cells can be isolated from peripheral blood mononuclear cells (PBMC) obtained from the host. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freez Clinical

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System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml of streptomycin (GIBCO). About  $2 \times 10^5$  cells in complete medium in a volume of, for example, 100  $\mu$ l are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). The antigen or peptides are added into the cultures in a final concentration of about 50  $\mu$ g/ml and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50  $\mu$ g/ml) on day 16.  $5 \times 10^5$  irradiated (4,000 rad) autologous PBMC are added in a volume of about 50  $\mu$ l complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human mIL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

#### Epitope mapping

The cytotoxic T-cells of the present invention can be used to determine the epitope of the carcinoma associate self-antigen that elicits a cytotoxic T-cell. For example, one can cut the antigen (protein) into numerous peptide fragments. Alternatively, the fragments can be chemically synthesized. Cytotoxic T-cells can then be plated and different fragments added to different wells. Only T-cells which recognize one of the pre-selected peptide fragments as an epitope will continue to expand, thereby permitting ready identification.

These fragments can then be used to elicit cytotoxic T-cell instead of using the whole protein. Additionally, one can prepare other



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fragments containing the epitope to enhance its ability to elicit a cytotoxic T-cell response. Modifications to these fragments are well known in the art and include the use of conjugates, specific amino acid residues such as cystines, etc.

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#### **Drug Assay**

The cytotoxic T-cell can also be used to screen for compounds which enhance the ability of the antigen to create a cytotoxic T-cell response. For example, cytotoxic T-cells can be incubated with a selected epitope, for example, in a microtiter plate. The compound to be tested, e.g. a drug, is then added to the well and the growth of the T-cells is measured. T-cell expansion indicates that the test compound enhances the T-cell response. Such compounds can be further evaluated.

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#### **Therapy**

The cytotoxic T-cell can be cultured to amplify its number and then injected back into the host by a variety of means. Generally, between  $1 \times 10^5$  and  $2 \times 10^{11}$  cytotoxic T-cells per infusion are administered in, for example, one to three infusions of 200 to 250 ml each over a period of 30 to 60 minutes. After the completion of the infusions, the patient may be treated with recombinant interleukin-2 with a dose of 720,000 IU per kilogram of body weight intravenously every eight hours; some doses can be omitted depending on the patient's tolerance for the drug. In addition, after infusion, additional antigen or fragments containing T-cell eliciting epitope(s) may be administered to the patient to further expand the T-cell number. The

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antigen or epitope may be formulated with an adjuvant and/or may be in a liposomal formulation.

5           The cytotoxic T-cells can also be modified by introduction of a viral vector containing a DNA encoding TNF and reintroduced into a host in an effort to enhance the anti-tumor activity of the cells. Other cytokines can also be used.

10           For parenteral administration, the recombinant vectors or the cytotoxic T-cells will typically be injected in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable parenteral carrier such as physiological saline.

15                           **REFERENCE EXAMPLE 1**  
                          **CONSTRUCTION OF VECTORS**

20           **Pox Viruses**

          A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., *Cell* 37:1053-1062 (1984); Morin et al., *Proc. Natl. Acad. Sci. USA* 84:4626-4630 (1987); Lowe et al., *Proc. Natl. Acad. Sci. USA*, 84:3896-3900 (1987); Panicali & Paoletti, *Proc. Natl. Acad. Sci. USA*, 79:4927-4931 (1982); Machett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415-7419 (1982)). Representative fowlpox and swinepox virus are available through the ATCC under accession numbers VR-229 and VR-363, respectively. A recombinant vaccinia - CEA is available  
30           through the ATCC under accession number VR2323.

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**DNA Vectors For *In Vivo* Recombination With A Parent Virus**

Genes that code for desired carcinoma associated antigens are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for *in vivo* recombination with a pox virus.

In general, the DNA donor vector contains the following elements:

- (i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;
- (ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);
- (iii) at least one gene encoding a desired protein located adjacent to a transcriptional promoter capable of directing the expression of the gene; and
- (iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in W091/19803, the techniques of which are incorporated herein by reference. In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments

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containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can be mono-di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

The donor vector preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al., *J. Virol.*, 62:1046 (1988); Falkner and Moss., *J. Virol.*, 62:1849 (1988); Franke et al., *Mol. Cell. Biol.*, 5:1918 (1985), as well as genes such as the *E. coli lacZ* gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al., *Gene*, 47:193-199 (1986)).

#### Integration Of Foreign DNA Sequences Into The Viral Genome And Isolation Of Recombinants

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques

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standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, WO89/03429).

5           Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing integrated DNA will be TK<sup>+</sup> and can be selected on this basis (Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415 (1982)). Alternatively, co-integration of a gene  
10           encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing  $\beta$ -galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al., *Gene*, 47:193  
15           (1986)).

          Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. The presence of integrated foreign DNA can be detected by hybridization with a labeled DNA  
20           probe specific for the inserted DNA. Preferred techniques for selection, however, are based upon co-integration of a gene encoding a marker or indicator gene along with the gene of interest, as described above. A preferred indicator gene is the *E. coli lacZ* gene which encodes the enzyme  $\beta$ -galactosidase. Selection of recombinant virus expressing  $\beta$ -  
25           galactosidase can be done by employing a chromogenic substrate for the enzyme. For example, recombinant viruses are detected as blue plaques in the presence of the substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -

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D-galactosidase or other halogenated-indolyl- $\beta$ -D-galactosidase (e.g., BluGal™).

#### **Characterizing The Viral Antigens Expressed By Recombinant Viruses**

5           Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme  
10           immunoassay (EIA).

#### **EXAMPLE I**

##### **Cytotoxic T-cell Response in Mouse Model**

#### **15           MATERIALS AND METHODS**

##### **Cells**

The MC-38 murine colonic adenocarcinoma cell line was supplied by the laboratory of Dr. S. Rosenberg (National Cancer Institute, Bethesda, MD). The derivative cell line expressing human CEA, designated MC-  
20           38-CEA-2, was developed by the transduction of the human CEA gene by the retroviral expression vector pBNC and has been previously described. Robbins, PF, et al., Cancer Res 1991; 51:3657-62.

##### **Recombinant baculovirus CEA (bV-CEA)**

25           Recombinant baculovirus CEA (bV-CEA) has been previously described as BVCEA-

140. Salgaller, ML, et al., *Cancer Res.* 1993;53:2154-61. bV-CEA encodes the full length human CEA gene and expresses several distinct

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molecular weight species ranging from 140 kDa to 110 kDa. These products reflect the genetic heterogeneity of the recombinant bV-CEA proteins due to homologous recombination between the three repeated domains of CEA. bV-CEA was purified from whole cell extract of the infected *Spodoptera frugiperda* cells as previously described. Salgaller, ML, et al., *Cancer Res.* 1993;53:2154-61. It has previously been shown that bV-CEA contains at least 10 CEA epitopes by reactivity to different monoclonal antibodies. Bei, R., et al., *Mole Immunolo* 1994;31:771-780. bV-CEA was shown to contain only simple high mannose carbohydrates as well as biantennary and biantennary hybrid carbohydrates, while native CEA also contains triantennary and tetraantennary complex sugars. Bei, R., et al., *Mole Immunolo* 1994; 31:771-780. bV-V has been previously described. Salgaller, ML, et al., *Cancer Res.* 1993;53:2154-61. It is the recombinant baculovirus containing only the shuttle vector with no CEA insert.

#### **Recombinant vaccinia CEA (rV-CEA)**

Recombinant vaccinia CEA was generated as previously described by Kantor, J., et al., *Cancer Res.* 1992;52:6917-25, the disclosure of which is herein incorporated by reference.

#### **Native CEA (nCEA)**

Commercially purified human CEA, designated nCEA for native CEA, was purchased from Vitro Diagnostic (Littleton, CO). It was derived from a liver adenoma.

#### **Immunization protocol**

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Six to eight weeks old female C57BL/6 mice were employed for the different immunization regimens. Animals were immunized either with purified native (nCEA) or recombinant CEA (bV-CEA). In each group of 10 animals, either, human CEA, purified bV-CEA or PBS alone in adjuvant was administered subcutaneously three times, at 14 day intervals. Each animal received 25  $\mu$ g of the purified protein for each immunization. The immunogens were emulsified in 25  $\mu$ g of monophosphoryl lipid A (MPL) + synthetic trehalose dicorynomycolate(TDCM) + cell wall skeleton (CWS) adjuvant (RIBI Immunochem Research, Inc., Hamilton, MT) in 200  $\mu$ l of phosphate buffered saline (PBS) (Biofluids Inc., Rockville, MD). Other groups of animals were primed with recombinant vaccinia CEA (rV-CEA) followed by immunization with the purified proteins. Ten animals in each group were inoculated by tail scarification with  $1 \times 10^7$  plaque forming units (pfu) of rV-CEA in 10  $\mu$ l of PBS. Subsequently the animals were boosted 14 days apart twice with 25  $\mu$ g of either nCEA, purified bV-CEA or PBS in adjuvant; that is RIBI adjuvant was used for all immunizations.

#### **Measurement of anti-CEA antibody titers.**

C57BL/6 female mice were immunized as described above. Serum was collected seven days after the last immunization and assayed for the presence of anti-CEA antibodies by ELISA (enzyme linked immunosorbent assay). Briefly, the bV-CEA as well as bV-V and the nCEA were diluted in PBS at 1  $\mu$ l of the mixture were incubated overnight at 37°C in polyvinylchloride microtiter plates (Dinatech, Chantilly, VA). Wells were treated with 5% bovine serum albumin (BSA) in PBS for 1 hr at 37°C and sera at different dilutions were



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added. Following 1 hr incubation at 37°C, the plates were washed with 1% BSA in PBS. Peroxidase coupled goat anti-mouse (Gibco BRL) was added followed by incubation for 1 hr at 37°C. After washing, ophenylene-diamine dihydrochloride was added in the presence of H<sub>2</sub>O<sub>2</sub>. After color development, the reaction was stopped with 50 µl of H<sub>2</sub>SO<sub>4</sub> and the absorbance read at 490 nm. The titer of the sera was defined as the dilution factor required to reach an optical density (O.D.) of 1.2.

#### 10      **Lymphoproliferation assay.**

C57BL/6 were immunized as described above to analyze induction of T cell activation by an assay measuring lymphocyte proliferation. One month following the last immunization, spleens were removed, mechanically dispersed through mesh screens and washed twice in serum free RPMI 1640 medium (Gibco, Gaithersburg, MD).

15      Erythrocytes and dead cells were removed by centrifugation over a Ficoll Hypaque gradient (density = 1.119 g/ml) (Sigma Chemical Co., St. Louis, Mo). Adherent cells were then depleted from the mononuclear population by incubation (30 min. at 37°C) and passage

20      over nylon wool columns (Robbins Scientific Corp., Sunnyvale, CA) resulting in a T cell enriched fraction. T lymphocytes were washed and resuspended in RPMI-1640 medium supplement with 15 mM HEPES (pH 7.4), 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL

25      streptomycin (all from Gibco BRL, Gaithersburg, MD) and 5 X 10<sup>-5</sup> β-mercaptoethanol (Sigma Chemical Co.).

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T lymphocytes ( $2 \times 10^5$ /well) were incubated in the presence of irradiated, normal syngeneic splenocytes ( $5 \times 10^5$ /well) as antigen-presenting cells with and without various stimuli, such as concanavalin A (ConA) (Sigma, Chemical Co.), nCEA (Vitro Diagnostic), bV-CEA, or purified ovalbumin (Sigma Chemical Co.) Incubation was carried out in 96-well flat bottom plates (Costar Corp., Cambridge, MA) for up to 3 days (ConA) or 5 days (antigens). Cultures were pulsed with  $^3\text{H}$ -thymidine ( $1 \mu\text{Ci}$ /well) (Du Pont/NEN Research Products, Wilmington, DE) for the final 18-24 hours of incubation. Cells were collected with a PhD cell harvester (Cambridge Technology, Cambridge, MA) and the incorporated radioactivity was measured by liquid scintillation spectroscopy (LS 3801 counter; Beckman Instruments, Duarte, CA)

#### **Prevention of growth of CEA transduced tumors (MC-38-CEA-2)**

Seven days following the last immunization,  $2 \times 10^5$  MC-38-CEA-2 tumor cells were transplanted by subcutaneous injection. Animals were checked weekly for the presence of tumor. Tumors were measured by caliper in two dimensions and the volumes were calculated using the formula  $(\text{width}^2 \times \text{length})/2$ .

#### **Results**

Mice were first evaluated for the ability of nCEA to act as a boost in mice previously administered rV-CEA. As mentioned in Materials and Methods, RIBI adjuvant was used in all injections in this study in which nCEA, bV-CEA or control PBS were used as immunogen. When mice were administered one administration of rV-CEA followed by two boosts of PBS antibody titers to nCEA were

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modest, i.e., 1:250. Similar titer was seen when the immune sera were tested against bV-CEA. When mice received one injection of rV-CEA followed by two administrations of nCEA, antibody titers were at least 10 fold higher to both nCEA (1:3,450) as well as bV-CEA (1:4,500). Previous studies have shown that when mice were given three administrations of nCEA, antibody titers averaged 1:1,600 against nCEA and 1:2,950 against bV-CEA. Studies were then undertaken to determine if the immunization scheme of one immunization of rV-CEA followed by two immunizations of nCEA could enhance specific T cell responses to CEA. Results of these experiments are given in Table 1, as T cell proliferative responses to nCEA, bV-CEA, as well as to control antigen ovalbumin and ConA. These results are also given as "lymphocyte stimulation induces" (LSI) in Table 2. The LSI is the  $^3\text{H}$ -thymidine incorporation of the test antigen divided by the  $^3\text{H}$ -thymidine incorporation of the control antigen ovalbumin. As can be seen in Tables 1 and 2, when mice were immunized three times with PBS, three times with nCEA, or one time with rV-CEA and two times with PBS, or one time with rV-CEA and two times with nCEA, the LSI values for ConA ranged from 110 to 240. When splenocytes of immunized mice were analyzed using 10  $\mu\text{g}/\text{ml}$  of nCEA, however, the proliferation response to one immunization of rV-CEA plus two immunizations of PBS was 1.9 versus an LSI value of 26.6 when mice received rV-CEA plus two immunizations of nCEA. It should also be noted that when splenocytes of immunized mice were tested against 100  $\mu\text{g}$  of nCEA the LSI of splenocytes of mice receiving three immunizations of nCEA was 39.6 versus 72.7 of mice receiving one injection of rV-CEA and two injections of nCEA. Similar results were seen when splenocytes of

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immunized mice were tested for proliferation responses using bV-CEA as an immunogen. These studies demonstrated that nCEA alone can elicit T cell responses to CEA and can also be used (perhaps more efficiently) in combination with rV-CEA to elicit specific anti-CEA T cell responses.

Studies were also carried out on the use of various immunization protocols to immunize mice against challenge with CEA expressing tumor cells. The transduction using a retroviral vector of the murine colon carcinoma cell line with CEA has been previously described. Robbins, PF, et al., *Cancer Res.* 1991;51:3657-62. In brief, these tumors express CEA and will grow in syngenic mice and kill transplanted animals. Horan Hand, P., et al., *Cancer Immunology Immunotherapy* 1993;36:65-75. As can be seen in Table 3, when mice received CEA transduced tumors following immunization with PBS, 7/9 mice had tumors at day 49 post transplant. When mice were given one administration of rV-CEA followed by two administrations of PBS, similar results were obtained, i.e., 7/10 mice had tumors. When mice were given either three administrations of nCEA, or one administration of rV-CEA followed by two administrations of nCEA, however, only 1/10 mice had tumors in both immunization groups.

Studies were then undertaken to determine if CEA derived from baculovirus could give similar results as those obtained with nCEA. Antibody titers of mice receiving one administration of rV-CEA followed by two administrations of bV-CEA averaged 1:14,800 against bV-CEA and were far in excess of the 1:250 average from mice receiving rV-CEA alone. Moreover, the induced antibodies were also shown to

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react with nCEA (1:8,600). As additional control in all these experiments, extracts of baculovirus devoid of the CEA gene (bV-V) were used and showed no reactivity. This control was employed to rule out the possibility that B cell responses were directed against baculovirus proteins as opposed to CEA specific responses. Previous studies have shown that when mice were given three administrations of bV-CEA, antibody titers averaged 1:3,100 against nCEA and 1:12,400 against bV-CEA. Bei, R., et al., *Mole Immunolo* 1994, 31:771-780.

As shown in Tables 4 and 5, lymphoproliferative responses and consequent LSIs were analyzed from splenocytes of mice receiving various immunization protocols of rV-CEA and/or bV-CEA. As shown in Tables 4 and 5, similar T cell proliferative responses were seen for all immunization groups to stimulation with ovalbumin or culture medium alone. Moreover, similar LSI results were seen in all immunization groups using ConA. As seen in Table 5, an LSI of 9.5 was seen using splenocytes of mice given three administrations of bV-CEA, after stimulation with 10  $\mu$ g/ml of bV-CEA. However, when mice were given one administration of rV-CEA and then two administrations of bV-CEA, T cell proliferation responses of 36.7 were seen. A similar enhancement of T cell response when immunizing with rV-CEA plus bV-CEA as opposed to three administrations of bV-CEA were also seen when using nCEA for stimulation. Whereas, lymphocytes from mice administered bV-CEA three times elicited lower LSI after stimulation with nCEA as compared to those stimulated by bV-CEA. Similar humoral and T lymphoproliferative responses were obtained when the

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same adjuvant devoid of TDCM was used to immunize mice with bV-CEA after priming with rV-CEA (data not shown).

5               Studies were then undertaken to determine whether various immunization protocols employing bV-CEA could be used to protect mice against challenge with CEA transduced tumors. As can be seen in Table 6, at both day 35 and day 49 time intervals, the immunization protocol using a single injection of rV-CEA followed by two immunizations of bV-CEA were more efficient than the use of three  
10              immunizations of bV-CEA in terms of both numbers of mice with tumors and average tumor volume.

              As shown in the studies described above, both bV-CEA and nCEA appear comparable in terms of inducing T cell responses and  
15              antibody responses either alone or in combination with rV-CEA. Moreover, both were able to protect mice against tumor challenge with CEA containing tumor cells when used in combination with rV-CEA. However, it should be pointed out that when bV-CEA was used alone as an immunogen the T cells proliferative response in vitro upon nCEA  
20              stimulation was lower as compared to those stimulated by bV-CEA and despite the high level of anti-nCEA antibodies. This result might be due to the differences of glycosylation between the two molecules.

Table 1

Lymphoproliferative responses of mice immunized with nCEA alone or nCEA after priming with recombinant vaccinia CEA.

Immunization Protocol  $^3\text{H}$  Thymidine incorporation, cpm

	Medium	ConA 2 $\mu\text{g/ml}$	OVA 100 $\mu\text{g/ml}$	nCEA		bV-CEA	
				10 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$
PBS(3)	1,213( $\pm$ 368) <sup>a</sup>	161,321( $\pm$ 7,899)	1,456( $\pm$ 159)	1,846( $\pm$ 280)	3,332( $\pm$ 2,252)	3,196( $\pm$ 506)	4,951( $\pm$ 508)
nCEA(3x)	727( $\pm$ 235)	139,980( $\pm$ 5,598)	1,147( $\pm$ 107)	N.A. <sup>b</sup>	45,454( $\pm$ 2,015)	11,990( $\pm$ 5,507)	19,459( $\pm$ 5,507)
rV-CEA(1X) + PBS(2X)	1,373( $\pm$ 442)	204,449( $\pm$ 7,704)	1,373( $\pm$ 451)	2,654( $\pm$ 257)	3,848( $\pm$ 736)	11,510( $\pm$ 454)	N.A
rV-CEA(1X) + nCEA(2X)	572( $\pm$ 284)	253,760( $\pm$ 57,540)	1,057( $\pm$ 594)	28,161( $\pm$ 7,132)	76,856( $\pm$ 3,814)	36,960( $\pm$ 1,480)	N.A

<sup>a</sup> Values = mean cpm  $\pm$  standard deviation of triplicate wells.

<sup>b</sup> Not Available

Table 2  
Lymphoproliferative responses of mice immunized with nCEA alone or nCEA  
after priming with recombinant vaccinia CEA.

Immunization Protocol	LYMPHOCYTE STIMULATION INDEX (LSI) <sup>a</sup>				
	ConA 2 µg/ml	OVA 100 µG/ML	nCEA 10 µg/ml	nCEA 100 µg/ml	bV-CEA 10 µg/ml 50µg/ml
PBS (3X)	110	1	1.2	2.2	2.1 3.4
nCEA(3X)	122	1	N.A. <sup>b</sup>	39.6	10.4 18.9
rV-CEA(1X) + PBS(2X)	148	1	1.9	2.8	8.4 N.A
rV-CEA(1X) + nCEA(2X)	240	1	26.6	72.7	34.9 N.A

<sup>a</sup> Proliferation in response to proteins is represented by an index of cpm in experimental wells divided by cpm of wells containing OVA 100 µg/ml.

<sup>b</sup> Not Available



Table 3

Effect of the immunization with nCEA alone or nCEA after priming with recombinant vaccinia CEA on the growth of MC-32-CEA-2 tumor cells that express human CEA.

Immunization Protocol	Day 35		Day 49	
	No. Mice with tumors/Total No. Mice	Average Volume (mm <sup>3</sup> )	No. Mice with tumors/Total No. Mice	Average Volume (mm <sup>3</sup> )
PBS(3X)	8/10	575	7/9*	1,990
nCEA(3X)	1/10	271	1/10	351
rV-CEA(1X) + PBS(2X)	7/10	690	7/10	3,574
rV-CEA(1X) + nCEA(2X)	1/10	974	1/10	7,096

\* Mice were immunized as described in Materials and methods. PBS or nCEA were administered in adjuvant. Seven days following the last immunization, mice were injected with 2X10<sup>5</sup> MC-38-CEA-2 tumor cells that express human CEA.

\* 1 animal died

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Table 4  
Lymphoproliferative responses of mice immunized with bV-CEA alone  
or bV-CEA after priming with recombinant vaccinia CEA.

Immunization Protocol  $^3\text{H}$  Thymidine incorporation, cpm

	Medium	ConA 2 $\mu\text{g/ml}$	OVA 100 $\mu\text{g/ml}$	nCEA		bV-CEA	
				10 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$
PBS(3X)	1,213( $\pm 366$ ) <sup>a</sup>	161,321( $\pm 7,899$ )	1,466( $\pm 159$ )	1,846( $\pm 280$ )	3,332( $\pm 2252$ )	3,196( $\pm 506$ )	4,951( $\pm 888$ )
bV-CEA(3x)	657( $\pm 78$ )	168,135( $\pm 11,975$ )	1,666( $\pm 751$ )	N.A. <sup>b</sup>	6,724( $\pm 2,015$ )	16,040( $\pm 3,578$ )	25,187( $\pm 3,728$ )
rV-CEA(1X) + PBS(2X)	1,373( $\pm 442$ )	204,449( $\pm 7,704$ )	1,373( $\pm 451$ )	2,654( $\pm 257$ )	3,848( $\pm 736$ )	11,510( $\pm 454$ )	N.A
rV-CEA(1X) + bV-CEA(2X)	1,141( $\pm 257$ )	185,186( $\pm 18,753$ )	1,846( $\pm 371$ )	28,634( $\pm 5,467$ )	31,634( $\pm 246$ )	67,774( $\pm 9,079$ )	N.A

<sup>a</sup> Values = mean cpm  $\pm$  standard deviation of triplicate wells.

<sup>b</sup> Not Available

Table 5  
Lymphoproliferative responses of mice immunized with nCEA alone  
or nCEA after priming with recombinant vaccinia CEA.

Immunization Protocol

LYMPHOCYTE STIMULATION INDEX (LSI)\*

	ConA 2 µg/ml	OVA 100 µg/ML	nCEA		bV-CEA	
			10 µg/ml	100 µg/ml	10 µg/ml	50 µg/ml
PBS (3X)	110	1	2.2	2.2	2.2	3.4
bVCEA (3X)	99.7	1	N.A. <sup>b</sup>	3.9	9.5	14.9
rV-CEA(1X) + PBS(2X)	148	1	1.9	2.8	8.4	N.A
rV-CEA(1X) + bVCEA(2X)	100	1	15.5	16.8	36.7	N.A

\*4 Proliferation in response to proteins is represented by an index of cpm in experimental wells divided by cpm of wells containing OVA 100 µg/ml.

<sup>b</sup> Not Available

Table 6

Effect of the immunization with nCEA alone or nCEA after priming with recombinant vaccinia CEA on the growth of MC-32-CEA-2 tumor cells that express human CEA.

Immunization Protocol	Day 35		Day 49	
	No. Mice with tumors/Total No. Mice	Average Volume (mm <sup>3</sup> )	No. Mice with tumors/Total No. Mice	Average Volume (mm <sup>3</sup> )
PBS(3X)	8/10	575	7/9*	1,990
bV-CEA(3X)	5/9	179	6/8*	972
rV-CEA(1X) + PBS(2X)	7/10	690	7/10	3,574
rV-CEA(1X) + bV-CEA(2X)	2/10	256	3/10	576

\* Mice were immunized as described in Materials and methods. PBS or bV-CEA were administered in adjuvant. Seven days following the last immunization, mice were injected with  $2 \times 10^5$  MC-38-CEA-2 tumor cells that express human CEA.

\* 1 animal died

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**EXAMPLE 2****Generation of human cytotoxic T-cells specific for the carcinoma self-associated antigen CEA**

5

**Materials and Methods****Cell Cultures**

Colorectal carcinoma cell lines: SW403 (HLA-A2, A3), HT-29 (HLA-A1, A9), SW837 (HLA-- , A19), SW1417 (HLA-A3,-) were purchased from American Type Culture Collection (Rockville, MD, USA). The cultures were mycoplasma free and were maintained in complete medium: DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO). The T2 cell line (transport deletion mutant) (28) was a kind gift from Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT, USA) and was maintained in Iscove's Modified Dulbecco medium (IMDM) containing 10% FBS.

20

EBV-transformed B cell lines designated as B-Vac24 and B-Vac01, and the B-Vac24 transfected with retroviral vector encoding a CEA construct designated as B-Vac24/CEA were maintained in RPMI1640 medium supplemented with 10% pooled human AB serum (Pel Freeze Clinical System, Brown Deer, WI, USA), 2mM glutamine, 100U/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO).

25

**Peptide Synthesis**

Peptide sequence of CEA was scanned for matches to the consensus motifs for HLA-A2 binding peptides. Nine, 10 and 11-mers

30

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peptides were selected for synthesis if they (a) fit the respective consensus motif and (b) diverged sufficiently from NCA and BGP so that an antigenic response might be anticipated. An NCA peptide that did not meet any known consensus motifs for human HLA-A2 was synthesized as a control. Syntheses were performed on an Applied Biosystem Model 432A personal peptide synthesizer and products were dissolved in aqueous solution, sterile-filtered and frozen at -70° at a concentration of 2 mg/ml. The purity of the peptides were >90% as analyzed by High Performance Liquid Chromatography (HPLC). The CEA peptides are listed in Table 7.

#### Peptide Binding Assay

Binding of CEA peptides to HLA-A2 molecule was analyzed by the upregulation of HLA-A2 expression of T2 cells as demonstrated by flow cytometry. T2 cell peptide binding assay has been reported recently. Nijman, H.W., et al. *Eur. J. Immunol.* 23:1215-1219, (1993). Briefly, aliquots of  $0.5-1 \times 10^6$  T2 cells in serum free IMDM were incubated with peptides at a concentration of 50  $\mu\text{g/ml}$  in 24-well culture plates at 37°C in 5% CO<sub>2</sub> overnight. T2 cells were washed and stained with an HLA-A2 specific antibody A2, 28 (#189 HA-1, One Lambda, Inc. Canoga Park, CA, USA) using 10  $\mu\text{l}$  of a 1x working dilution/ $10^6$  cells. MOPC-104E (Cappel/Organon Teknika Corp., West Chester, PA) was used as isotype control. The cells were then washed three times and incubated with a 1:100 dilution of phycoprobe PE anti-mouse IgM (Biomedica Corp., Foster City, CA). Analysis was carried out using the FACScan as above. Cells were maintained on ice during all cell preparation and staining unless otherwise stated above.

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#### Introduction of CEA Gene in EBV-Immortalized B-Cell lines

B-cell lines were generated by a standard method Blumberg, R.S., et al., *J Infect Dis* 155: 877-880, (1987) using the B95-8 marmoset cell line supernatant containing EBV. Human pooled AB serum was used in all cell cultures in this study. EBV-immortalized B-cell lines were transduced with a retroviral expression construct of CEA. Robbins, P.F., et al. *Cancer Res* 51: 3657-3662, (1991). Transduction was performed by co-cultivation of EBV-immortalized B cells with productively transduced amphotropic retroviral packaging cell lines PA317-CEA as described by Tsang et al. Tsang, K.Y., et al., *J Immunother* 13: 143-152, (1993). EBV immortalized B-cell transductants were selected in medium containing G418 at an active concentration of 0.7 mg/ml.

#### Generation of T Cell Line (TCL)

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from patients with metastatic carcinoma in a Phase I clinical trial employing recombinant vaccine in which the CEA gene was inserted into an attenuated vaccinia virus (rV-CEA). Kantor, J., et al. *J Natl Cancer Inst* 84: 1084-1091, (1992); Kantor, J., et al. *Cancer Res* 52: 6917-6925, (1992). PBMC were obtained prior to and post administration of three injections of rV-CEA at monthly intervals at  $10^5$  pfu (Vac 7),  $10^6$  pfu (Vac 6), and  $10^7$  pfu (Vac 24) per injection. PBMC from patients were separated using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described. Boyum, A., *Cand J. Clin. Lab Invest* 21:77-80 (1968). Washed PBMC were resuspended in complete medium: RPMI 1640 (GIBCO) supplemented with 10% pooled human AB serum (Pel-Freez

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Clinical System, Brown Deer, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml of streptomycin (GIBCO).  $2 \times 10^5$  cells in complete medium in a volume of 100  $\mu$ l were added into each well of 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA).

5 Peptides were added to cultures at a final concentration of 50  $\mu$ g/ml. Cultures were incubated for 5 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After removal of the peptide containing medium, the cultures were then provided with human IL-2 (10 U/ml) for 11 days, with IL-2 containing medium being replenished  
10 every 3 days. The 5 day peptide plus 11 day IL-2 incubations constitute one cycle. Primary cultures were restimulated with the same peptide (50  $\mu$ g/ml on day 16 to begin the next cycle.  $5 \times 10^5$  irradiated (4,000 rad) autologous PBMC were added in a volume of 50  $\mu$ l in complete medium as antigen-presenting cells (APC).

#### Cytotoxicity Assays

15 Various target cells were labeled with 50  $\mu$ Ci of 111-Indium (<sup>111</sup>In) oxine (Medi-Physics Inc., Arlington, IL, USA) for 15 min. Target cells ( $0.5 \times 10^4$ ) in 100  $\mu$ l were added to each of 96 wells in U-bottom  
20 assay plates (Costar). The labeled targets were incubated with peptides at a final concentration of 50  $\mu$ g/ml for 60 min at 37°C in CO<sub>2</sub> before adding effector cells. Effector cells were suspended in 100  $\mu$ l of complete medium supplemented with 10% pooled human AB serum and added to target cells, and the plates were then incubated at 37°C  
25 in 5% CO<sub>2</sub> for 12 to 18 hr. Supernatant was harvested for gamma counting with use of Skatron Harvester frames (Sterling, VA, USA). Experiments were carried out in triplicate. Specific lysis was calculated with the use of the following formula:



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$$\text{Lysis\%} = \frac{\text{observed release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

5 Spontaneous release was determined from wells to which 100  $\mu$ l of complete medium were added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

#### Detection of Cytokine

10 Supernatants of T cells exposed for 3 days to peptides and APC in IL-2 free medium at a responder to stimulator ratio of 4:1 ( $4 \times 10^6$ :  $10^6$  cells/ml) were screened for the secretion of TNF- $\alpha$  using enzyme-linked immunosorbent assay kit (Genzyme, Cambridge, MA, USA). The results were expressed in pg/ml.

#### Flow Cytometry

15 The procedure for single color flow cytometric analysis has been previously published. Guadagni et al., *Cancer Res.* 50:6248-6254 (1990). Briefly,  $1 \times 10^6$  cells were washed three times with cold  $\text{Ca}^{2+}$ -free Dulbecco's PBS (DPBS) and then stained for 1 hr with 1  $\mu$ g of MAb against CD3 (Beckton Dickinson, San Jose, CA), CD4 (Becton Dickson), CD8 (Becton Dickinson), HLA class I (W6/32) (Sera-Lab, Sussex, England), HLA class II (DR) (Beckton Dickinson), and MOPC-21 (Cappel/Organon Teknika Corp., West Chester, PA) in a volume of 100  $\mu$ l of PBS containing 1% bovine serum albumin. Anti-CEA MAb COL-1 was used as 100  $\mu$ l of culture supernatant. The cells were then washed three times with cold DPBS and incubated for an additional hr in the presence of 1:100 dilution (volume of 100  $\mu$ l of PBS containing

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1% bovine serum albumin) of fluorescein-conjugated goat anti-mouse Ig (Kirkegaard and Perry Laboratory, Gaithersburg, MD). The cells were again washed three times with DPBS and resuspended in DPBS at a concentration of  $1 \times 10^6$  cells/ml. The cells were immediately analyzed using a Beckton Dickinson FACScan equipped with a blue laser with an excitation of 15nW at 488nm. Data were gathered from 10,000 cells using a live gate, stored, and used to generate results.

The procedure for dual color flow cytometric analysis was similar to that for single color analysis with the following exceptions. The antibodies used were anti-CD4 fluorescein conjugate, anti-CD8 phycoerythrin conjugate, anti-IgG<sub>1</sub> fluorescein conjugate, and anti-IgG<sub>2a</sub> phycoerythrin conjugate (Becton Dickinson). Staining was done simultaneously for 1 hr after which cells were washed three times, resuspended as above, and immediately analyzed using a Becton Dickinson FACSort equipped with a blue laser with an excitation of 15nW at 488 nm equipment with Lysis™II program.

#### HLA Typing

The HLA phenotyping of patients was kindly performed by the Tissue Typing QC Laboratory, Naval Medical Research Institute using a standard antibody-dependent microcytotoxicity assay and a defined panel of anti-HLA antisera. The HLA phenotypes were as follows: Patient Vac24 [HLA-A2,24 (9); B 44 (12,w4), 51 (5,W4); DR 4,11 (5); DQ 3,7 3); DR w52,53]; Patient Vac01 [HLA-A28,31; B14,35; DR1, 4; DQ1,3; Drw53]; Patient Vac23 [HLA-A1, 26 (10); B8 (w6), 60 (40,w6); CW3,7; DR0103, 15 (2); DQ5 (1),6(1)]; Patient Vac32 [HLA-A3, 68(28); B7 (w6),51 (5,w4); CW7; DR4, 15(2); DQ1,8(3); DRw53];

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patient Vac6[HLA-A2,24(9); B13(W4); CW6; DR7, 8; DQ4; DR53], an patient Vac7 [HLA-A2; B7(W6); CW7: DR15(2), 17(3); DQ1,2; DR52].

#### Vaccinia Virus Infection of Colorectal Carcinoma Cells

5 cDNA for HLA-A2.1 gene and human  $\beta$ 2-microglobulin gene in vaccinia virus vector were obtained from Dr. D. Cole (Surgery Branch, NCI, NIH). These genes were inserted into the TK gene in plasmid pSCII, allowing homologous recombination to occur with the viral TK gene. O'Neil, B.H., et al. *J Immunol* 151: 1410-1418, (1993). Target  
10 cells at a concentration of  $1 \times 10^7$ /ml in complete RPMI1640 medium supplemented with 0.1% BSA were incubated with an equal volume of vaccinia virus ( $10^8$  plaque-forming unit/ml) in the same medium at 37°C for 1.5 hr. The cells were then adjusted to a concentration of  $5 \times 10^5$ /ml in complete medium and incubated for 3 hr at 37°C.  
15 Coinfection of vaccinia-HLA-A2 and vaccinia- $\beta$ 2-microglobulin was done at a multiplicity of infection of 10:1.

#### Statistical Analysis

Statistical analysis of differences between means was done by a  
20 paired T-test.

#### **Results**

Since the entire amino acid sequence of human CEA is known and human HLA class I A2 consensus motifs have been described  
25 [Falk, K., et al., *Nature* 351: 290-296 (1991); Hunt, D.F., et al., *Science* 255: 1261-1263, (1992)], studies were undertaken to identify a series of peptides that would potentially bind class I A2 molecules. A2 was chosen since it is the most common HLA class I molecule,

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being represented in approximately 50% of North American caucasians and 34% of African Americans. Lee J., *Springer-Verlag, New York* 6: 154, (1990). The peptide sequence of CEA was thus examined for matches to the consensus motifs for HLA-A2 binding peptides.

5 Peptides were only selected, moreover, if their sequence diverged sufficiently from the CEA-related NCA and BGP sequences. The amino acid sequence of human CEA (GeneBank Accession #M17303) was scanned using a predictive algorithm [Parker, K.C., et al., *J Immunol* 152: 163-175, (1994)] that combines a search for anchor residues  
10 with numerical assignments to all residues at all positions. Ten peptides were synthesized using this algorithm, ranging in length from 9 to 11 amino acids. Six of these peptides also contained the HLA-A2 motif of leucine or isoleucine at position 2 and valine or leucine at the C terminal. Another peptide (CAP-7) also possessed the motif for  
15 binding to HLA-A3. DiBrino, M., et al., *Proc Natl Acad Sci* 90: 1508-1512, (1993). All peptides were selected to have minimal homology to the parallel regions of NCA and BGP, after optimal alignment of the latter sequences with CEA. The 9 mer, 10 mer or 11 mer peptides that met these criteria were selected for synthesis and purification;  
20 they were designated CAP (Carcinoembryonic Antigen Peptide)-1 through 10 (SEQ ID NOS: 1-10). Their amino acid sequence and position in the CEA molecule are given in Table 7. The positive (P) or negative (N) designation (Table 7) relates to the predicted binding to HLA-A2.

25 The T2 cell binding assay has been used to predict human HLA-A2 consensus motifs. Nijman, H.W., et al., *Eur. J. Immunol.* 23:1215-1219 (1993). In this assay, the binding of an appropriate

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peptide results in the upregulation of surface HLA-A2 on the T2 cells, which can be quantified via FACScan using an anti HLA-A2 antibody. As seen in Table 7, seven of the CEA peptides (CAP-1 through CAP-7) scored positive for T2 binding (the peptides were designated CAP-1 through 10 retrospectively on the basis of their quantitative binding to T2). Since peptide 571-579 (designated CAP-1) demonstrated the highest level of T2 binding, the peptide reflecting the NCA analog (the corresponding NCA peptide obtained after optimal alignment of NCA and CEA), was also synthesized and tested; this peptide, designated NCA-1, showed background binding to T2 (Table 7). This was consistent with the fact that an amino acid substitution in NCA had abolished one of the A2 anchor residues (Arg for Leu at position 2).

In an attempt to establish T-cell lines from patients who had received the rV-CEA construct, PBLs were obtained from three patients (designated Vac6, Vac7, and Vac24) who express the HLA-A2 allele and alternately pulsed with 50µg/ml peptide CAP-1 and IL-2 (10U/ml) as described in the Methods section. In all three cases T-cell lines could be established which were cytotoxic for T2 cells when pulsed with CAP-1 peptide. Figure 1 shows the results of these assays using T-cell lines from patients V24 and V6. The T-cell line from patient Vac24 was chosen for further study.

PBLs from patient Vac24 (pre-and post-vaccination with 3 doses of 10<sup>7</sup> pfu rv-CEA at monthly intervals) were placed in 96 well plates and pulsed with the CAP-1 peptide and then IL-2 as described in the Methods section. Each exposure to peptide and IL-2 was considered one cycle

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of stimulation. As seen in Table 8, one, two, or three cycles of CAP-1 peptide and IL-2 did not result in growth of cells in any of the 96 wells using the pre-immunization PBLs. By contrast after one cycle of stimulation of post-vaccination PBLs from the same patient, 66 of the 96 wells (68%) demonstrated growth of cells, which were maintained through four cycles of stimulation. It is of interest, that after four cycles of stimulation of pre-immunization PBSs, 2/96 wells (2%) exhibited cell growth. Thus, one could hypothesize that a minor population of T-cells exist in this patient capable of recognizing a specific CEA epitope (571-579), and that these cells were clonally expanded as a result of the rV-CEA administration.

Sufficient PBSs pre-and post-vaccination with rV-CEA at the  $10^7$  pfu dose, were also available from two non-HLA-A2 patients: Vac32(HLA A1,26) and Vac23 (HLA A3,68). Since we had little or no basis for predicting which peptides might bind to these haplotypes, nine of the CEA peptides were used in an attempt to establish T-cell lines. Using peptide CAP-1 with IL-2 as described above, no T-cell lines could be established from pre-immunization PBLs from either patient Vac32 or Vac23 (Table 9). However, employing post-rV-CEA immunization PBLs, T-cell lines were established after 3 cycles of stimulation in 25/48 wells (52%) for patient Vac32 and in 21/48 wells (43%) for patient Vac23 (Table 9).

A similar contrast in pre- versus post-vaccination PBLs from patients Vac32 and Vac23 was seen with a mixture of CEA peptides CAP-4, 6 and 7 (Table 9). Combinations were used as an initial screen to conserve PBLs. It is perhaps of significance that PBL from

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Vac32 (HLA-A3 positive) showed evidence of cell growth in the presence of CAP-7, the peptide that bears the HLA-A3 binding motif. It should be noted that these results suggest that a peptide shown to bind to HLA-A2 can also stimulate T-cell lines after binding to some non-A2 antigens; the possible reasons for this will be discussed in detail below. However, it was decided to first characterize the T-cell responses in patient Vac24 because of the implied relevance of MHC binding and T-cell activation. Nonetheless, it is encouraging that PBL from 5/5 patient showed signs of T-cell response to peptide CAP-1 post immunization with rV-CEA.

Flow cytometric studies were conducted to phenotype the V24T, V6T and V7T-cell lines. The results are shown in Table 10. Cells stained double positive for both CD8 and CD4 and V24T and V6T cell lines, while the V7T cell line was CD8<sup>+</sup>.

To determine if the Vac24 T-cell line (designated V24T) could lyse autologous B-cells presenting the CAP-1 peptide, B-cells from patient Vac24 were first transformed with EBV and then pulsed (i.e., incubated) with the CAP-1 peptide. As seen in Table 11, the V24T-cells were capable of lysing the autologous B-cells when pulsed with CAP-1, but when an allogeneic (non-HLA-A2) EBV-transformed B-cell was pulsed with the same peptide, no lysis was observed. When the NCA-1 peptide, reflecting the analogous region on the NCA molecule was used to pulse Vac24 B-cells, no lysis was observed with the V24T-cells. As shown in Table 7, this was not unexpected since 3 of the 9 amino acids of NCA-1 differ from those of CAP-1, including an anchor residue.

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Studies were undertaken to determine if the CAP-1 peptide could induce the secretion of TNF- $\alpha$  from the cytolytic V24T-cells. As shown in Figure 2, incubation of V24T-cells with autologous B-cells pulsed with CAP-1 peptide resulted in the production of substantial TNF- $\alpha$ , while control peptides CAP-9 and CAP-10 failed to show this effect.

While the above results indicate that autologous B-cells present the CAP-1 peptide to the Vac24 cells, resulting in lysis of the B-cells, they do not indicate that human APCs can endogenously process the entire CEA molecule in a manner so as to bind HLA-A2 molecules for presentation at the cell surface. To help answer this question, EBV transformed B-cells of patient Vac24 were transduced with the entire human CEA gene using a retroviral vector (see Methods). As seen in Table 12, the CEA-transduced cells now express CEA, and the transduction process had no effect on expression of (cells bearing) HLA class I and class II molecules.

As shown in Table 13, the autologous B-cells transduced with the CEA gene can now serve as targets for the V24 CTLs. These results thus demonstrate that a CEA gene product can be endogenously processed by autologous B-cells and presented at the cell surface in the context with class I MHC to induce T-cell lysis. The question now remained as to whether human carcinoma cells can act in the same manner as APCs and thus serve as potential targets for V24T-cells. As seen in Table 13, non-A2 allogenic carcinoma cells SW1417 and HT-29, which do express substantial CEA cannot serve as targets while the allogeneic A2 positive SW403 carcinoma cells expressing CEA are lysed.



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To further demonstrate the HLA-A2 restricted nature of the V24T-cells in the lysis of human carcinoma cells, the CEA positive, non-A2 SW837 human carcinoma cell line was employed. These cells were either uninfected, infected with wild type vaccinia virus, or infected with recombinant vaccinia virus containing the HLA-A2 gene. As seen in Table 14, only the carcinoma cells infected with rV-A2.1 recombinant expressed A2, and only these cells were susceptible to lysis with V24T cells. These studies further demonstrate the HLA-A2 restricted nature of the CEA specific lysis of the V24T-cells.

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**Table 7**  
**Binding of CEA peptides to HLA-A2 molecule**

Peptide	Position	Sequence	Predicted Binding The HLA Class 1A2 <sup>a</sup>	T2 binding Assay <sup>b</sup>	
				Exp 1	Exp2
CAP-1	571-579	YLSGANLNL (SEQ ID NO:1)	P	561	806
CAP-2	555-564	VLYGPDTPII (SEQ ID NO:2)	P	515	796
CAP-3	87-96	TLHVIKSDLV (SEQ ID NO:3)	P	480	ND
CAP-4	1-11	KLTIESTPFNV (SEQ ID NO:4)	P	441	ND
CAP-5	345-354	TLLSVTRNDV (SEQ ID NO:5)	P	405	ND
CAP-6	19-28	LLVHNL PQHL (SEQ ID NO:6)	P	381	ND
CAP-7	27-35	HLFGYSWYK (SEQ ID NO:7)	N	326	ND
CAP-8	523-532	TLFNVTRNDA (SEQ ID NO:8)	N	260	ND
CAP-9	137-146	TQDATYLWWV (SEQ ID NO:9)	N	204	ND
CAP-10	102-110	GQFRVYPEL (SEQ ID NO:10)	N	201	ND
NCA-1	571-579	YRPGENLNL (SEQ ID NO:11)	N	252	225
Positive Control	--	ALAAAAAAV (SEQ ID NO:12)	P	632	--
No Peptide	--	--	--	280	300

<sup>a</sup>Predicted binding on the basis of published motifs. Folk, et al., *Nature* 351:290-296 (1991).

<sup>b</sup>Reactivity of T2 cells with anti-HLA-A2 Mab after the cells were incubated with CEA peptide. Peptides were used in concentration of 50  $\mu$ g/ml/10<sup>6</sup> cells. The results are expressed in relative fluorescence values.

Positive control 9-mer is an A2 binding motif.

**Table 8**  
**Frequency of T Cell Growth from Patient Vac24 Following an In Vitro Stimulation**  
**with CAP-1 Peptide: Pre- and Post Immunization with rV-CEA**

Pre or Post Immunization	Stimulation cycle			
	1 <sup>a</sup>	2	3	4
Pre	0 <sup>b</sup>	0	0	2
Post	66	66	66	66

<sup>a</sup>cycle of stimulation. See Material and Methods for details with CEA-CAP-1, 9 mer peptide an IL-2.

<sup>b</sup>Results were expressed as the number of positive wells/96 wells.

PBMC were seeded at a concentration of  $1 \times 10^5$  cell/200 $\mu$ l/well.

**Table 9**  
**Frequency of T Cell Growth Following an In Vitro Stimulation with CEA Peptides:**  
**Pre and Post Immunization with rV-CEA**

Patient	Peptide	Frequency					
		Pre			Post		
		1 <sup>a</sup>	2	3	1	2	3
VAC32	CAP-4, CAP-6, CAP-7 CAP-3, CAP-10, CAP-9 CAP-8, CAP-2 CAP-1	0 <sup>b</sup>	0	4	0	0	30
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	25
VAC23	CAP-4, CAP-6, CAP-7 CAP-3, CAP-10, CAP-9 CAP-8, CAP-2 CAP-1	0	0	3	0	0	31
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	21

<sup>a</sup>cycle of stimulation with peptide IL-2 (see Material and Methods).

<sup>b</sup>Results were expressed as the number of positive wells/48 wells. PBMC were seeded at a concentration of  $1 \times 10^6$  cells/well in 1 ml.

**TABLE 10**  
**FLOW CYTOMETRIC ANALYSIS OF SURFACE MARKERS ON T-CELL LINES**

Surface antigen	V24T	V6T	V7T
CD8/CD4	62.4	30.8	Negative
CD8	35.7	56.0	99.6
CD4	Negative	12.6	Negative
CD3	98.5	95.4	99.4

Negative, <5% positive. Results are expressed in percentage of each T-cell line reactive with MABs. Routinely 2-4% of the cells were stained when treated with either with no primary MAB or an isotype-matched control MAB.

Table 11

Ability of V24T-cells (T-cells derived from PBLs from rV-CEA Vaccinated Patient) to Lyse Autologous and Allogenic B-Cells Pulsed with a CEA Specific Peptide

B-Cells	HLA-A2	Pulsing Peptide	% Lysis
B-V24	+	CAP-1	<b>43% (0.29)</b>
		NCA-1	<b>8% (1.92)</b>
		none	<b>2% (0.37)</b>
B-V01	-	CAP-1	<b>10% (0.53)</b>
		NCA-1	<b>11% (0.62)</b>
		none	<b>8% (0.68)</b>

A 18hr<sup>111</sup>In release assay was performed. Peptides were used in a concentration of 50µg/ml. Results are expressed as percentage specific lysis at E/T ratios of 25:1. Number in parenthesis is standard deviation.

Bold number, statistically significant ( $p < 0.01$ )(paired t-test).

**Table 12**

**Flow Cytometric Analysis of Surface Antigens of EBV Transformed  
B-cells derived from Patient Vac24 (designated B-Vac24):  
Before and After Transfection with CEA**

Antigen	MAB	% Positive	
		B-VAC24 <sup>a</sup>	B-Vac24 (CEA) <sup>b</sup>
CEA	COL-1	4.4(24.7) <sup>c</sup>	42.9(100.9)
HLA class I	W6/32	100.0(831.9)	100.0(519.1)
HLA class II	anti-HLA-DR	99.8(313.3)	99.3(221.8)
Control	MOPC-21	2.2(30.5)	2.0(24.1)

<sup>a</sup>B-Vac24 are EBV transformed B-cells derived from PBL of patient Vac 24 prior to immunization with rV-CEA.

<sup>b</sup>B-Vac24 (CEA) are the same as B-Vac24 except they have been transduced with the entire human CEA gene using a retroviral vector as described in Materials and Methods.

<sup>c</sup>Values represent the percentage of each cell type reactive with MABs listed as analyzed by flow cytometry. Numbers in paranthesis are the mean channel fluorescence intensity as determined in relative log units. Routinely 2-4% of the cells were stained when treated either with no primary MAB or an isotype-matched control MAB. LS174T, a colorectal carcinoma cell line, was used as a positive for CEA expression. The % positive value for CEA in CEA expressing LS174T human colon carcinoma cell line was 59.8 (144.4).

**Table 13**  
**Cytotoxicity of V24T-Cell Line (derived from a patient immunized with rV-CEA)**  
**on Target Cells with Endogenous CEA Expression**

Target	HLA-A2	CEA	% Lysis
B-VAC24 <sup>a</sup>	+	-	8.2 (2.1)
B-VAC24 (CEA) <sup>a</sup>	+	+	<b>46.1 (11.6)</b>
SW403 <sup>b</sup>	+	+	<b>45.2 (1.5)</b>
SW1417 <sup>b</sup>	-	+	5.2 (0.5)
HT-29 <sup>b</sup>	-	+	4.1 (0.6)

<sup>a</sup>As described in the legend to Table 11.

<sup>b</sup>Human colon carcinoma cell lines expressing CEA.

HLA-A2 and CEA expression were tested by flow cytometry using MAbs anti-A2 and COL-1 respectively. A 18 hr<sup>11</sup>In release assay was performed. Results are expression in % specific lysis at E:T ratio = 50:1. The number in parentheses is the standard derivation. Bold numbers, statistically significant lysis ( $p < 0.01$ )(paired t-test).



**Table 14**  
**Demonstration of HLA-A2 involvement in Ability of V24T Cells**  
**to Lyse Human Colon Carcinoma Cells**

Infection	Expression of MHC class 1 molecules <sup>a</sup>		% Lysis <sup>b</sup>
	W6/32	A2.28	
None	99.1 (228.23)	4.4 (47.41)	8.2 (1.4)
Vaccinia A2.1	99.3 (261.75)	90.4 (66.70)	31.4 (2.5)
Vaccinia vector control	99.4 (217.59)	2.9 (17.54)	7.2 (0.6)

SW837, an HLA-A2 negative and CEA positive, human colorectal carcinoma cell line was infected with vaccinia A2.1. The surface expression of HLA-A2 molecule after infection was analyzed by flow cytometry using anti-HLA-A2 MAb and W6/32 antibody.

<sup>a</sup> Values represent the percentage of cells reactive with the antibodies listed. Number in parentheses are the mean fluorescence intensity as determined in relative log units.

<sup>b</sup> Results are expressed in % specific lysis in a 12-hr <sup>111</sup>In release assay at E:T ratio = 50:1. The number in parentheses is the standard deviation. Bold numbers, statistically significant lysis (p < 0.01)(paired t-test).

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This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

5

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Schlom, Jeffrey  
Panicali, Dennis

(ii) TITLE OF INVENTION:  
GENERATION OF HUMAN CYTOTOXIC T-CELLS  
SPECIFIC FOR CARCINOMA SELF-ASSOCIATED ANTIGENS  
AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 12

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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- (A) TELEPHONE: (617) 523-3400
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Leu Ser Gly Ala Asn Leu Asn Leu  
1                      5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Leu Tyr Gly Pro Asp Thr Pro Ile Ile  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Leu His Val Ile Lys Ser Asp Leu Val  
1                    5                    10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Leu Thr Ile Glu Ser Thr Pro Phe Asn Val  
1                    5                    10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Leu Leu Ser Val Thr Arg Asn Asp Val  
1                    5                    10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Leu Val His Asn Leu Pro Gln His Leu  
1                   5                   10

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

His Leu Phe Gly Tyr Ser Trp Tyr Lys  
1                   5

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Thr Leu Phe Asn Val Thr Arg Asn Asp Ala  
1                   5                   10

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr	Gln	Asp	Ala	Thr	Tyr	Leu	Trp	Trp	Val
1			5			10			

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly	Gln	Phe	Arg	Val	Tyr	Pro	Glu	Leu
1			5					

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Tyr	Arg	Pro	Gly	Glu	Asn	Leu	Asn	Leu
1			5					

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid

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(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Leu Ala Ala Ala Ala Ala Val  
1                      5



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What is claimed is:

1. A method for producing human cytotoxic T-cells specific for a carcinoma self-associated antigen comprising:
  - a) introducing a sufficient amount of a first pox virus vector to a host to stimulate production of cytotoxic T-cells, wherein the pox virus vector has at least one insertion site containing a DNA segment encoding the carcinoma self-associated antigen or an epitope thereof operably linked to a promoter capable of expression in the host; and
  - b) at at least one periodic interval thereafter, contacting the host with additional antigen.
2. The method of claim 1, wherein the host is contacted with the additional antigen by introducing a second pox virus vector to the host having at least one insertion site containing a DNA segment encoding the carcinoma self-associated antigen or an epitope thereof operably linked to a promoter capable of expression in the host.
3. The method of claim 1, wherein the carcinoma self-associated antigen is carcinoembryonic antigen.
4. The method of claim 1, wherein the epitope comprises a peptide selected from the group of peptides represented in the Sequence Listing as SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

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5. The method of claim 1, wherein the epitope comprises a peptide represented in the Sequence Listing as SEQ ID NO: 1.
6. The method of claim 1, wherein the pox virus is selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus.
7. The method of claim 6, wherein the orthopox virus is vaccinia.
8. The method of claim 5, wherein the avipox is fowlpox, canary pox and pigeon pox.
9. The method of claim 5, wherein the suipox is swinepox.
10. The method of claim 2, wherein the first pox virus vector is vaccinia and the second pox virus vector is selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus.
11. A method for isolating a human cytotoxic T-cells specific for a carcinoma self-associated antigen comprising:
  - a) introducing a sufficient amount of a first pox virus vector to a host to stimulate production of cytotoxic T-cells, wherein the pox virus vector has at least one insertion site containing a DNA segment encoding the carcinoma associated antigen or an epitope thereof operably linked to a promoter capable of expression in the host;

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- b) at at least one periodic interval thereafter, contacting the host with additional antigen; and
- c) isolating cytotoxic T-cells specific for carcinoma associated antigens from the host.

12. The method of claim 11, further comprising:

- d) expanding the number of T-cells by contacting the isolated cells of step c) alternately with the carcinoma self-associated antigen or an epitope thereof and IL-2.

13. A method for mapping an epitope of a carcinoma self-associated antigen comprising:

- a) introducing a sufficient amount of a first pox virus vector to a host to stimulate production of cytotoxic T-cells, wherein the pox virus vector has at least one insertion site containing a DNA segment encoding the carcinoma self-associated antigen operably linked to a promoter capable of expression in the host;
- b) at at least one periodic interval thereafter, contacting the host with additional antigen;
- c) isolating cytotoxic T-cells specific for carcinoma self-associated antigens from the host;

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d) contact the T-cells of step c) with pre-selected epitopes and selecting T-cells recognizing said epitopes.

14. A method for producing human cytotoxic T-cells specific for carcinoma self-associated antigen comprising introducing a T-cell eliciting epitope of the carcinoma self-associated antigen to a host.

15. The method of claim 14, wherein additional T-cell eliciting epitope is introduced to the host at at least one periodic interval thereafter.

16. The method of claim 15, wherein the T-cell eliciting epitope is formulated with an adjuvant or is in a liposomal formulation.

17. The method of claim 16, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

18. The method of claim 14, wherein the carcinoma self-associated antigen is CEA.

19. The method of claim 14, wherein the T-cell eliciting epitope is selected from the group consisting of peptides represented in the Sequence Listing as SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

20. The method of claim 19, wherein the T-cell eliciting epitope is a peptide represented in the Sequence Listing as SEQ ID NO: 1.

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21. A method for isolating a human cytotoxic T-cell specific for a carcinoma self-associated antigen comprising introducing a T-cell eliciting epitope of the carcinoma self-associated antigen to a host and isolating cytotoxic T-cells specific for carcinoma associated antigens from the host.

22. The method of claim 21, further comprising expanding the number of T-cells by contacting the isolated cytotoxic T-cells alternately with the carcinoma self-associated antigen or an epitope thereof and IL-2.

23. The method of claim 21, wherein the T-cell eliciting epitope is formulated with an adjuvant or is in a liposomal formulation.

24. The method of claim 23, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

25. The method of claim 21, wherein the carcinoma self-associated antigen is CEA.

26. The method of claim 25, wherein the T-cell eliciting epitope is selected from the group consisting of peptides represented in the Sequence Listing as SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

27. The method of claim 26, wherein the T-cell eliciting epitope is a peptide represented in the Sequence Listing as SEQ ID NO: 1.

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28. A method for treating a host having a tumor expressing a carcinoma self-associated antigen comprising introducing cytotoxic T-cells specific for the antigen to the host and at at least one periodic interval thereafter introducing to the host a T-cell eliciting epitope of the carcinoma self-associated antigen.

29. The method of claim 28, wherein the T-cell eliciting epitope is formulated with an adjuvant or is in a liposomal formulation.

30. The method of claim 29, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

31. The method of claim 28, wherein the carcinoma self-associated antigen is CEA.

32. The method of claim 31, wherein the T-cell eliciting epitope is selected from the group consisting of peptides represented in the Sequence Listing as SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

33. The method of claim 32, wherein the T-cell eliciting epitope is a peptide represented in the Sequence Listing as SEQ ID NO: 1.

34. A peptide selected from the group consisting of peptides represented in the Sequence Listing as SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

Figure 1A

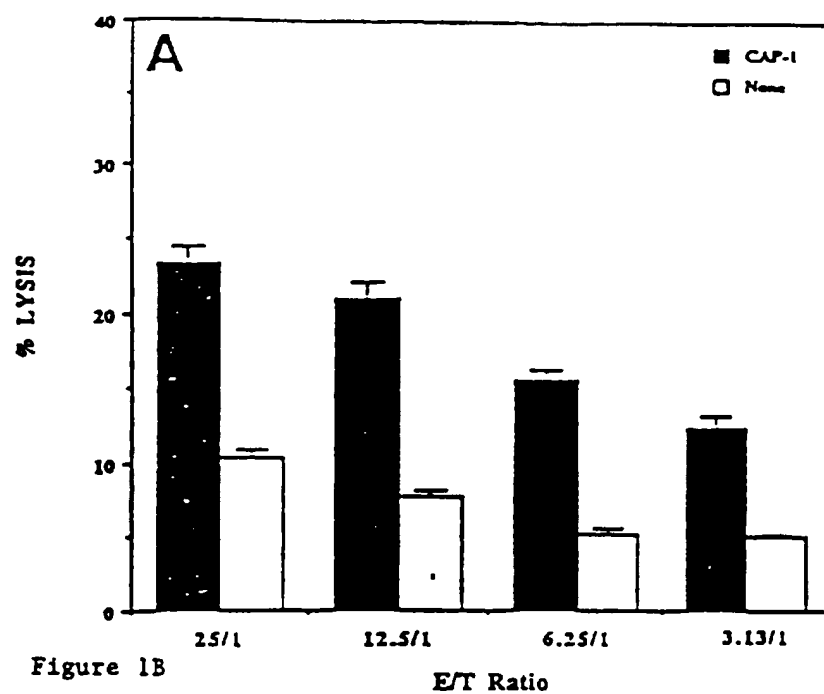


Figure 1B

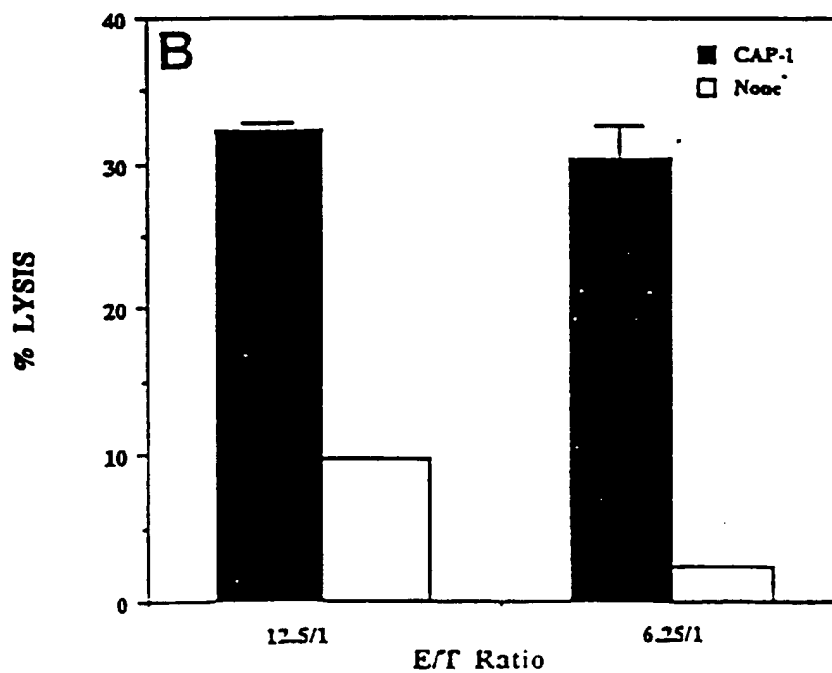
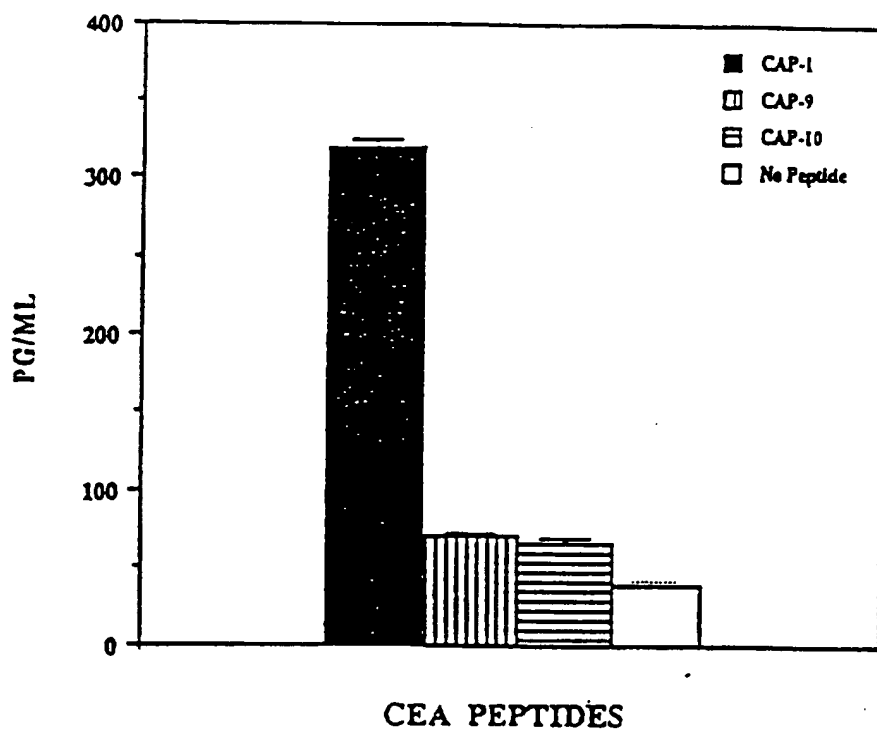


Figure 2





## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/02156

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12N15/12 A61K39/00 A61K35/14 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>JOURNAL OF THE NATIONAL CANCER INSTITUTE,          vol. 84, 1992,          pages 1084-1091, XP000577052          KANTOR ET AL: "ANTITUMOR ACTIVITY AND          IMMUNE RESPONSES INDUCED BY A RECOMBINANT          CARCINOEMBRYONIC ANTIGEN-VACCINIA VIRUS          VACCINE"          cited in the application          see page 1084, abstract; page 1086, last          paragraph-page 1087, first paragraph          ---          -/--</p>	<p>1-3,6,7,          10-18,          21-25,          28-31</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

2 August 1996

Date of mailing of the international search report

07. 08. 96

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Sitch, W

## INTERNATIONAL SEARCH REPORT

International Application No

PLI/US 96/02156

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CANCER RESEARCH, vol. 52, 1992, pages 6917-6925, XP002009982 KANTOR ET AL: "IMMUNOGENICITY AND SAFETY OF A RECOMBINANT VACCINIA VIRUS VACCINE EXPRESSING THE CARCINOEMBRYONIC ANTIGEN GENE IN A NONHUMAN PRIMATE" cited in the application see page 6917,abstract;page 6920,paragraph 2;page 6921,paragraph 2-page 6922,paragraph 1</p> <p>---</p>	<p>1-3,6,7, 10-18, 21-25, 28-31</p>
X	<p>WO,A,92 19266 (US ARMY) 12 November 1992</p> <p>see page 3, line 8 - line 28 see page 8, line 34 - page 11, line 21</p> <p>---</p>	<p>1-3,6,7, 10-18, 21-25, 28-31</p>
X	<p>AIDS RESEARCH AND HUMAN RETROVIRUSES, vol. 10, no. S1, 1994, page S58 XP002009983 SCHLOM ET AL: "RECOMBINANT VACCINES FOR THE ACTIVE SPECIFIC IMMUNOTHERAPY OF HUMAN CANCER" see abstract</p> <p>---</p>	<p>1-3,6,7, 10-18, 21-25, 28-31</p>
X	<p>EP,A,0 343 946 (HOPE CITY) 29 November 1989 see page 5, line 44 - line 47; tables 1,2 see page 6, line 44 - line 45</p> <p>---</p>	<p>34</p>
X	<p>EP,A,0 263 933 (MOLECULAR DIAGNOSTICS INC) 20 April 1988 see claims 1,2,7</p> <p>---</p>	<p>34</p>
A	<p>CANCER RESEARCH, vol. 53, 1 May 1993, pages 2154-2161, XP002009984 SALGALLER ET AL: "BACULOVIRUS RECOMBINANTS EXPRESSING THE HUMAN CARCINOEMBRYONIC ANTIGEN GENE" cited in the application</p> <p>---</p>	
A	<p>NATURE, vol. 351, 23 May 1991, pages 290-296, XP002009985 FALK ET AL: "ALLELE-SPECIFIC MOTIFS REVEALED BY SEQUENCING OF SELF-PEPTIDES ELUTED FROM MHC MOLECULES" cited in the application</p> <p>---</p>	

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## INTERNATIONAL SEARCH REPORT

International Application No

PLI/US 96/02156

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BREAST CANCER RESEARCH AND TREATMENT, vol. 38, no. 1, 1996, pages 27-39, XP000578043 SCHLOM ET AL: "STRATEGIES FOR THE DEVELOPMENT OF RECOMBINANT VACCINES FOR THE IMMUNOTHERAPY OF BREAST CANCER" see the whole document ---	1-3,6,7, 10-18, 21-25, 28-31
P,X	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 982-990, XP000567117 TSANG ET AL: "GENERATION OF HUMAN CYTOTOXIC T CELLS SPECIFIC FOR HUMAN CARCINOEMBRYONIC ANTIGEN EPITOPES FROM PATIENTS IMMUNIZED WITH RECOMBINANT VACCINIA-CEA VACCINE" see the whole document ---	1-34
P,X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 36, March 1995, page 249 XP002009986 TSANG ET AL: "A RECOMBINANT CEA-VACCINIA VACCINE INDUCES A CEA-SPECIFIC CYTOTOXIC T-CELL RESPONSE IN CARCINOMA PATIENTS" see abstract 1483 ---	1-3,6,7, 10-18, 21-25, 28-31
P,X	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 949-951, XP000566533 MITCHELL: "T-CELL-MEDIATED IMMUNITY TO CARCINOEMBRYONIC ANTIGEN IN HUMANS:AN EXAMPLE OF 'SWIMMING UPSTREAM'?" see the whole document ---	1-3,6,7, 10-18, 21-25, 28-31
E	WO,A,96 14074 (UNIV CALIFORNIA) 17 May 1996 see page 1, line 16 - line 23 see page 31, line 10 - line 19 see page 33, line 4 - page 34, line 10 see page 41, line 8 - page 42, line 13 see page 49, line 27 - page 51, line 27 see page 56, line 1 - page 58, line 2 -----	14,18, 21,23,25

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 02156

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-10, 14-20, 28-33 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound /composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/02156

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9219266	12-11-92	AU-B- 2006092	21-12-92
		CA-A- 2102623	07-11-92
		EP-A- 0584266	02-03-94
		JP-T- 6508025	14-09-94
-----			
EP-A-0343946	29-11-89	AU-B- 3515589	30-11-89
		JP-A- 2107189	19-04-90
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EP-A-0263933	20-04-88	AU-B- 598573	28-06-90
		AU-B- 7686887	24-03-88
		DE-D- 3787864	25-11-93
		DE-T- 3787864	10-02-94
		ES-T- 2059330	16-11-94
		IE-B- 60279	29-06-94
		US-A- 5231009	27-07-93
		US-A- 5274087	28-12-93
		JP-A- 63119681	24-05-88
		US-A- 5122599	16-06-92
		ZA-A- 8705951	19-02-88
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WO-A-9614074	17-05-96	AU-B- 4198196	31-05-96
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